# A Homogeneous, High Throughput Assay to Measure Guanine Nucleotide Exchange Factor Activity Using the Transcreener® GDP Assay

**Meera Kumar and Robert G. Lowery**
**BellBrook Labs, Madison, WI**


## INTRODUCTION

Guanine nucleotide exchange factors (GEFs) positively regulate Rho GTPases by accelerating GDP dissociation to allow formation of the active, GTP-bound complex. Development of inhibitors that specifically disrupt GEF action on a target Rho GTPase may be a promising therapeutic strategy for blocking Rho-dependent oncogenesis. Unfortunately, identification of GEF inhibitors has been painfully slow, in part, because the available GEF assays are not well suited for high throughput screening (HTS). To overcome these technical hurdles, we have developed a simple, generic biochemical assay for measuring GEF activity based on the fact that GDP dissociation is generally the rate-limiting step in the GTPase catalytic cycle, and thus, the addition of GEF causes an increase in steady-state GTPase activity. The increase in GDP production is determined using Transcreener® GDP assay, which relies on selective fluorescence polarization (FP) based immunodetection of GDP. As a proof of concept, we have tested the applicability of this method to selected combinations of Rho family GTPases (Cdc42, RhoA, RhoB, Rac1) and GEFs (Dbs, P-Rex1). Dbs caused dose-dependent increases in GTPase activity for Cdc42, Rho A, and Rho B with maximal GEF effects of 5.1-, 2.8-, and 16.3-fold, respectively; P-Rex1 increased the GTPase activity of Rac1 by as much as 14-fold. In addition, we carried out a 6,400 compound pilot screen of small molecules from a diversity library at the Chemical Genomics Center at the Lankenau Institute for Medical Research (LIMR) formatted in an orthogonally pooled format for the P-Rex1/Rac1 pair. We observed a hit rate of 0.33%, with a signal window of ~100 mP and Z' of 0.71, indicating the robustness of the assay with sufficient signal magnitude and precision for HTS. It is noteworthy that unlike fluor-GTP binding assays, this method is based on multiple catalytic cycles for both the GEF and the GTPase with an unmodified GTP substrate; thus, it is a more direct measure of GEF's intrinsic functional activity. In summary, the Transcreener® GDP assay provides a HTS-compatible and mechanistically unbiased assay that can be used for diverse GTPase/GEF pairs.

## ASSAY PRINCIPLE

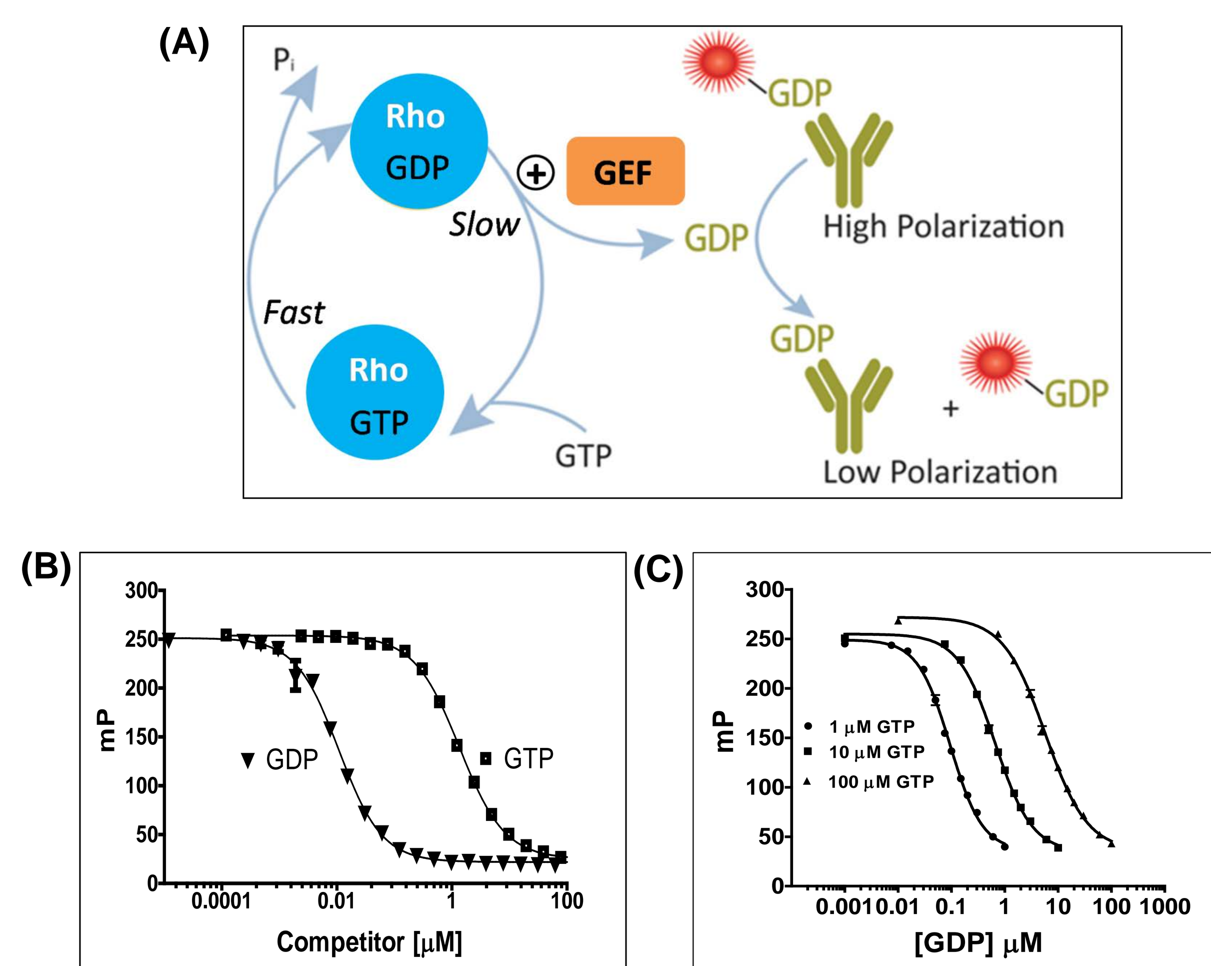



**Figure 1. GEFs accelerate steady-state GTP hydrolysis rates. (A)** By accelerating the rate limiting step of the GTPase catalytic cycle, GEFs enhance the steady state rates of GDP formation by GTPases. These changes can be detected using the Transcreener® GDP assay, which relies on highly selective antibodies and far red fluorescent tracers to allow homogeneous detection of GDP with fluorescence polarization (FP) readout. **(B)** Greater than 100-fold selectivity enables detection of GDP in the presence of excess GTP. **(C)** Standard curves mimicking the enzymatic conversion of GTP to GDP. The dynamic range of the assay can be easily tuned for different initial GTP concentrations.

## GTPase AND GEF CONCENTRATIONS

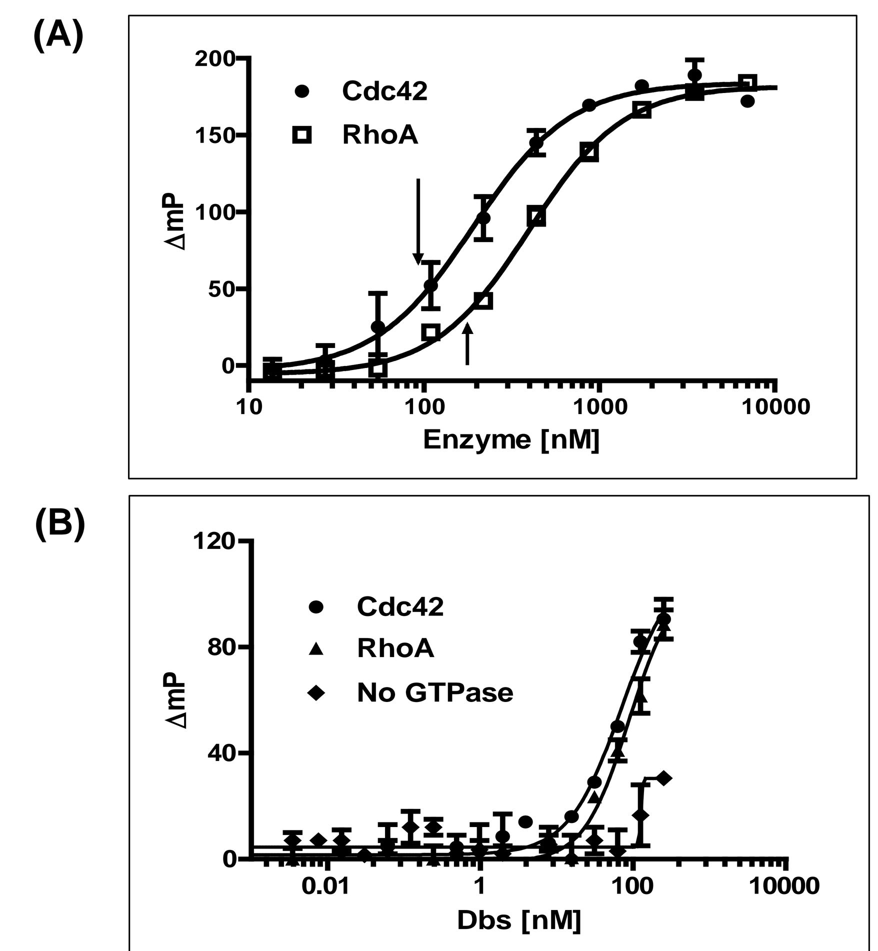



**Figure 2. Determination of optimal GTPase and GEF concentrations: (A)** GTPases (Cdc42 and RhoA) are first titrated to identify a concentration that produces ~20% of the maximal signal. The data indicate that 39 nM Cdc42 and 78 nM RhoA would be optimal for detecting GEF-dependent stimulation of steady-state GTPase activity. **(B) Titration of Dbs with limiting GTPase to determine optimal Dbs concentration**. When Dbs was added to reactions containing a low concentration of RhoA and Cdc42, a dose-dependent increase in the signal was observed, which suggests that accelerating GDP dissociation would increase the steady-state rate of GTPase activity. The optimal Dbs concentration was 100 nM.

## EFFECT OF Dbs ON Cdc42, RhoA, AND RhoB ACTIVITY

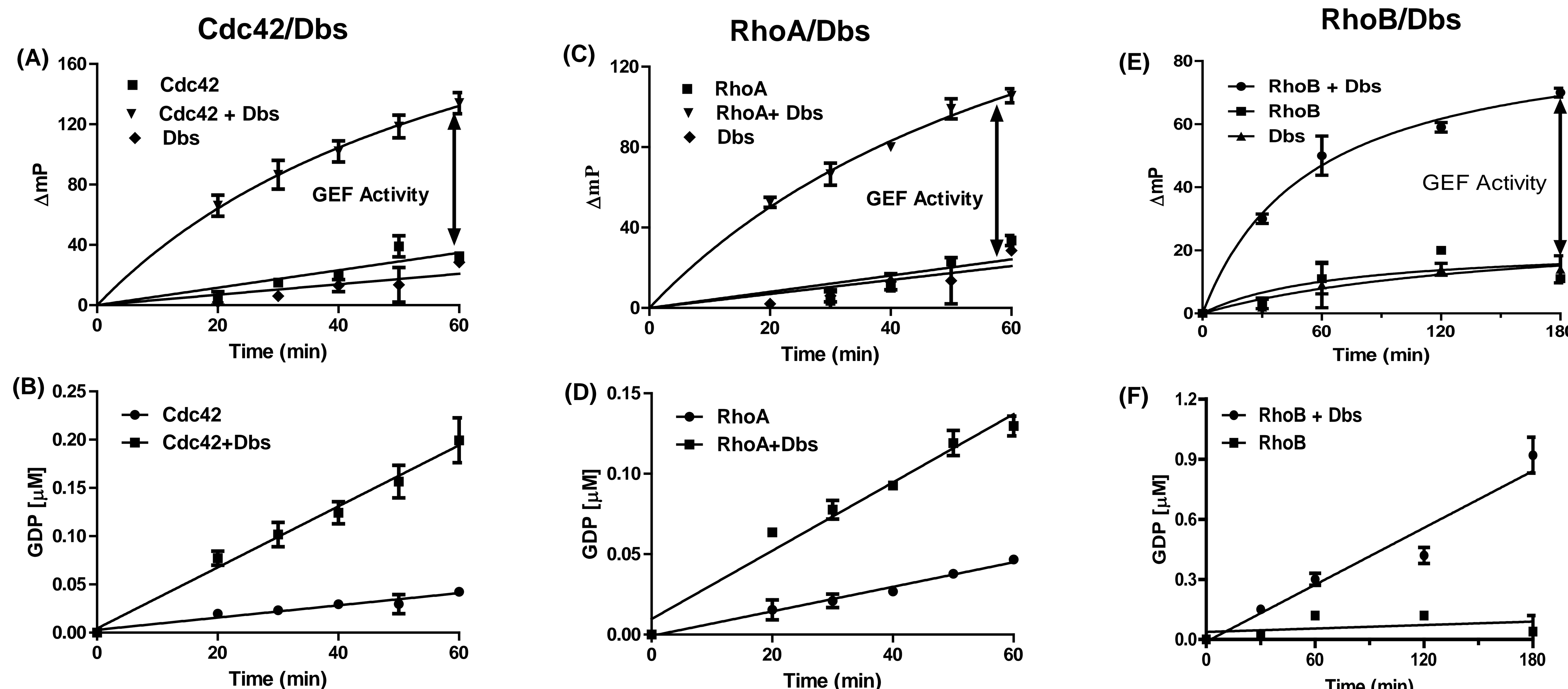
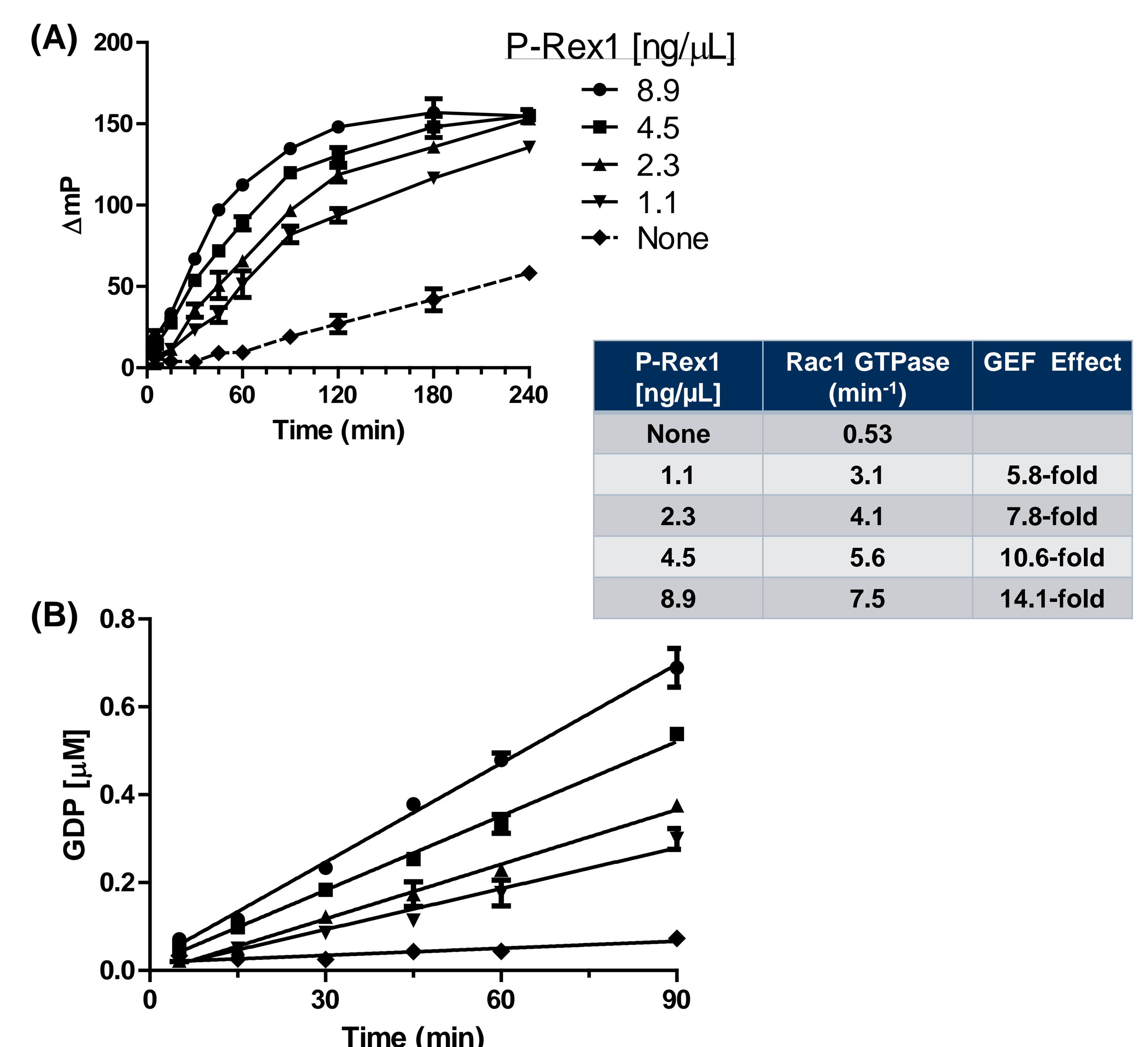



**Figure 3. GEF effect of Dbs on Rho GTPases.** Time-course data showing the change in GTPase activity of **(A)** CDC42, **(C)** RhoA, and **(E)** RhoB in the presence and absence of Dbs, as measured by changes in FP using Transcreener® GDP assay. The polarization values from the GTPase/GEF reactions were converted to GDP concentrations **(B,D,F)** using a GDP/GTP standard curve (Figure 1C). **Table**: GEF effect on GTPase activity was quantified using the ratio of GTPase activity with and without Dbs.

| | GTPase rate (min⁻¹) | GEF Effect |
|---|---|---|
| Cdc42 | $1.62 \times 10^{-2}$ | |
| Cdc42 + Dbs | $8.21 \times 10^{-2}$ | 5.1-fold |
| RhoA | $9.70 \times 10^{-3}$ | |
| RhoA + Dbs | $2.69 \times 10^{-2}$ | 2.8-fold |
| RhoB | $7.25 \times 10^{-4}$ | |
| RhoB + Dbs | $1.18 \times 10^{-2}$ | 16.3-fold |

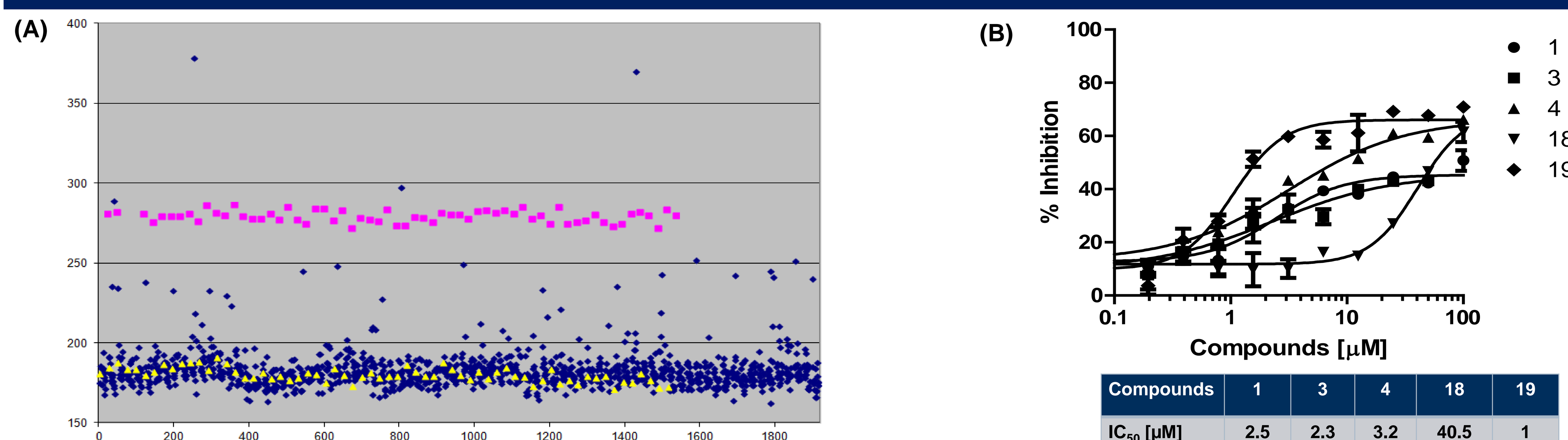
## GEF ACTIVITY OF P-Rex1 WITH Rac1




| P-Rex1 [ng/μL] | Rac1 GTPase (min⁻¹) | GEF Effect |
|---|---|---|
| None | 0.53 | |
| 1.1 | 3.1 | 5.8-fold |
| 2.3 | 4.1 | 7.8-fold |
| 4.5 | 5.6 | 10.6-fold |
| 8.9 | 7.5 | 14.1-fold |

**Figure 4. P-Rex1 stimulates Rac1 GTPase activity**. Reactions containing a limiting concentration of Rac1 (50 nM), 10 µM GTP, and Transcreener® GDP detection reagents were titrated with P-Rex1 (DH-PH domain) and read at regular intervals. **(A)** Time-course for P-Rex1 dependent stimulation of Rac1 GTPase activity. **(B)** FP data converted to GDP concentration using standard curve (Figure 1C). **Table**: Rates of GDP formation by Rac1 in the presence of varying concentrations of P-Rex1.

## PILOT SCREEN OF P-Rex1/Rac1




| Compounds | 1 | 3 | 4 | 18 | 19 |
|---|---|---|---|---|---|
| $IC_{50}$ [µM] | 2.5 | 2.3 | 3.2 | 40.5 | 1 |

**Figure 5. HTS assay for identification of P-Rex1 inhibitors. (A)** Using the Transcreener® GDP assay, we carried out a 6,400 compound pilot screen of small molecules from a diversity library at the Chemical Genomics Center at the Lankenau Institute for Medical Research (LIMR) formatted in an orthogonally pooled format (10x, n=2). The signal window was approximately 100 mP, Z' was 0.71, and the hit rate was 0.33%. Data is presented as a scatterplot with signal (mP) on the y-axis and well number on the x-axis. Uninhibited controls (no compound) are yellow and 100% inhibited controls (no P-Rex1) are magenta. **(B) Dose response curves.** Excluding artifacts, 7 compounds exhibited dose-dependent inhibition of P-Rex1/Rac1 reaction. Of these, 2 were excluded as Rac1 inhibitors. Table shows the $IC_{50}$ value of these compounds.

## CONCLUSIONS

- Measuring stimulation of steady-state GTP hydrolysis is a viable approach for detecting Rho-GEF activity.
- We demonstrated the suitability of the Transcreener® GDP assay to measure the GEF effect of Dbs on Cdc42, RhoA, and RhoB, as well of P-Rex1 on Rac1.
- The dynamic range of the assay can be easily tuned for different initial GTP concentrations and GTPase/GEF pairs.
- The Transcreener® GDP Assay can be used for screening and profiling for GEF inhibitors in an HTS-compatible format.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BellBrook Labs, 5500 Nobel Drive, Suite 230, Madison, WI 53711
866.313.7881 or 608.443.2400